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FLEXIBILITY OF COUPLING AND STOICHIOMETRY OF ATP FORMATION IN INTACT CHLOROPLASTS*

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SUMMARY

Since coupling between phosphorylation and electron transport cannot be measured directly in intact chloroplasts capable of high rates of photosynthesis, attempts were made to determine ATP/2 e ratios from the quantum requirements of glycerate and phosphoglycerate reduction and from the extent of oxidation of added NADH via the malate shuttle during reduction of phosphoglycerate in light. These different approaches gave similar results. The quantum requirement of glycerate reduction, which needs 2 molecules of ATP per molecule of NADPH oxidized was found to be pH-dependent. 9–11 quanta were required at pH 7.6, and only about 6 at pH 7.0. The quantum requirement of phosphoglycerate reduction, which consumes ATP and NADPH in a 1/1 ratio, was about 4 both at pH 7.6 and at 7.0. ATP/2 e ratios calculated from the quantum requirements and the extent of phosphoglycerate accumulation during glycerate reduction were usually between 1.2 and 1.4, occasionally higher, but they never approached 2.

Although the chloroplast envelope is impermeable to pyridine nucleotides, illuminated chloroplasts reduced added NAD via the malate shuttle in the absence of electron acceptors and also during the reduction of glycerate or CO_2. When phosphoglycerate was added as the substrate, reduction of pyridine-nucleotides was replaced by oxidation and hydrogen was shuttled into the chloroplasts to be used for phosphoglycerate reduction even under light which was rate-limiting for reduction. This indicated formation of more ATP than NADPH by the electron transport chain. From the rates of oxidation of external NADH and of phosphoglycerate reduction at very low light intensities ATP/2e ratios were calculated to be between 1.1 and 1.4.

Fully coupled chloroplasts reduced oxaloacetate in the light at rates reaching 80 and in some instances 130 μmoles · mg^{-1} chlorophyll · h^{-1} even though ATP is not consumed in this reaction. The energy transfer inhibitor phlorizin did not significantly suppress this reduction at concentrations which completely inhibited photosynthesis. Uncouplers stimulated oxaloacetate reduction by factors ranging

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Abbreviations: P_i, inorganic phosphate; FCCP, carbonylcyanide *p*-trifluorophenylhydrazone.

[*] Dedicated to Dr R. Hill, Cambridge, on the occasion of his 75th birthday.

from 1.5 to more than 10. Chloroplasts showing little uncoupler-induced stimulation of oxaloacetate reduction were highly active in photoreducing CO_2. Measurements of light intensity dependence of quantum requirements for oxaloacetate reduction gave no indication for the existence of uncoupled or basal electron flow in intact chloroplasts. Rather reduction is brought about by loosely coupled electron transport. It is concluded that coupling of phosphorylation to electron transport in intact chloroplasts is flexible, not tight. Calculated ATP/2e ratios were obtained under conditions, where coupling should be expected to be optimal, i.e. at low phosphorylation potentials [ATP]/[ADP] [P_i]. Flexible coupling implies, that ATP/2e ratios should decrease with increasing phosphorylation potentials inside the chloroplasts.

INTRODUCTION

The consumption of ATP during photosynthetic reduction of CO_2 to the sugar level in the Calvin cycle is higher by a factor of 1.5 or more than oxidation of NADPH [1]. On the basis of early measurements indicating that only 1 ATP is formed during reduction of NADP by the electron transport chain of chloroplasts (cf. ref. 2) it was assumed that the extra ATP needed was provided by cyclic electron flow [3]. However, attempts to demonstrate a contribution of cyclic photophosphorylation to photosynthetic CO_2 reduction have failed [4, 5]. There is in fact considerable doubt as to whether significant rates of cyclic electron flow are possible in vivo in the presence of oxygen [6]. Recent measurements by Hall and others have indicated that not one but 2 molecules of ATP can be synthesized during transfer of 2 electrons from water to a suitable acceptor molecule such as ferricyanide [7–10]. Evidence has also accumulated that two coupling sites exist in the electron transport chain of chloroplasts [11–13]. However, a stoichiometry of ATP formation in relation to NADP production of 2/1 would, in the face of a requirement of 1.5/1, not be much less of a problem than that of 1/1 envisaged earlier. ATP would accumulate during CO_2 reduction since side reactions such as protein synthesis are not fast enough to drain sufficient ATP. High ATP levels are known to inhibit electron flow [9, 14]. Thus ATP production in excess of consumption should actually lead to inhibition of photosynthesis. This is clearly not what is observed. Indeed, indirect measurements have shown that in intact chloroplasts which are capable of efficient photosynthesis, considerably less than 2 molecules of ATP are synthesized during the transport of two electrons to NADP [5]. In this communication attempts to determine coupling ratios in intact chloroplasts are described. This is not possible by direct measurements of ATP formation in relation to electron flow, since intact chloroplasts have a low permeability to adenylates.

MATERIAL AND METHODS

Chloroplasts were isolated from greenhouse or field-grown spinach following a modification [5] of Jensen and Bassham's method [15] immediately after illuminating freshly picked leaves for about 5 min at 0 °C with a 500 W lamp from a distance of 50–80 cm. Oxygen evolution was recorded by a Clark type electrode. The medium for glycerate, 3-phosphoglycerate, oxaloacetate and CO_2 reduction contained 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM ethylene diamine tetraacetate, 10 mM NaCl, 0.5 mM inorganic phosphate and 50 mM *N*-2-hydroxypiperazine-*N*-2-ethane sulfonate (HEPES), pH 7.6 (occasionally pH 7.0). During CO_2 reduction 3 mM dithiothreitol was also present. Substrate concentrations were usually 2 mM. The same medium was also used for measuring transfer of reducing equivalents across the chloroplast envelope by the malate shuttle [17]. Further additions were in this case 50 mM malate, 0.4 mM NAD, 20 μg/ml malate dehydrogenase (EC 1.1.1.37, Boehringer, Mannheim) and NADH as specified. CO_2-free medium was prepared by degassing reaction medium under vacuum at pH 4 and subsequent flushing with nitrogen. Chloroplasts were added equivalent to 33 or 66 μg of chlorophyll per ml for oxygen electrode work and to 40 μg per ml for spectrophotometric recordings of NAD reduction or NADH oxidation by the malate shuttle. For the measurement of quantum yields, the absorption of 674 nm light (interference filter 674 nm, half band width 12 nm, 1mm Calflex C, from Balzers, Liechtenstein, 3 mm RG 630 from Schott, Mainz, and 5–8 cm of water) by the chloroplast suspension was measured in a large Ulbricht sphere. The chloroplast suspension was in the same lucite reaction cuvette that was used for recording evolution. Saturating red light for photoreactions of chloroplasts was produced by 1 mm Calflex C, 3 mm RG 630 and 8 cm of water. Light-dependent pyridine nucleotide reduction or oxidation by chloroplasts was excited by short wavelength red light (1 mm Calflex C, 3 mm RG 610, broad band interference filter K6 from Balzers, Liechtenstein, 6 mm heat absorbing glass and 8 cm water, half band width from 625–685 nm). The photomultiplier measuring absorption at 340 nm was protected by an interference filter 340 nm, a Corning filter 5840 and a Schott filter UG1. During the reaction the intensity of actinic light was recorded by a silicone photodiode, which was calibrated by a compensated thermopile (Kipp and Zonen, Delft). Reactions were performed at 20 °C. Activities were corrected for broken chloroplasts which absorbed light without contributing to the reactions. The percentage of intact chloroplasts in the suspensions was routinely measured by the ferricyanide method [16] and ranged from 70–90 % in different isolations. K^+ contents were measured by flame photometry and Mg^{2+} contents by atomic absorption.

RESULTS AND DISCUSSION

1. Quality of the chloroplasts

The chloroplasts used for this study reduced CO_2 at rates comparable to those exhibited by the parent leaves. CO_2 reduction of chloroplasts from actively growing leaves ranged between 80 and 230 μmoles · mg^{-1} chlorophyll · h^{-1} at light saturation. Chloroplasts from old leaves exhibited lower rates and were rarely used. Loss of activity was usually below 20 % after 5 h storage at 0 °C. In addition to bicarbonate, the chloroplasts reduced at high rates added 3-phosphoglycerate (140–220/μmoles · mg^{-1} chlorophyll · h^{-1}) and at still significant rates glycerate [21] (20–60 μmoles · mg^{-1} chlorophyll · h^{-1}), oxaloacetate (20–130 μmoles · mg^{-1} chlorophyll · h^{-1}) and nitrite (5–20 μmoles · mg^{-1} chlorophyll · h^{-1}). Reduction of the latter two compounds was increased by uncoupling with 10 mM NH_4Cl. Reduction rates of glycerate were lower than those of phosphoglycerate because of rate-limiting levels of glycerate kinase in the chloroplast stroma. The chloroplasts retained a high concentration of Mg^{2+} (approx. 25 mM) and of K^+ (approx. 20 mM), which did not leak out. They exhibited fluorescence phenomena indicative of light-dependent cation fluxes between stroma and thylakoid compartments [18], which are similar to those shown by intact leaves (Kautsky phenomenon) [19]. It is concluded that the chloroplasts were completely functional photosynthetically.

2. Quantum requirement of glycerate reduction

The reduction of 3-phosphoglycerate by intact chloroplasts has recently been shown to require 4–5 quanta of 674 nm light [5]. This is in agreement with current ideas about cooperation between two photosystems [20]. In contrast, the reduction of one molecule of CO_2 needed more than 12 quanta [5]. During phosphoglycerate reduction one molecule of NADPH is oxidized and one molecule of ATP hydrolyzed. Intact chloroplasts can also reduce added glycerate [21]. After its uptake glycerate is phosphorylated by ATP. The subsequent reduction of the resulting phosphoglycerate (PGA) gives rise to oxygen evolution according to

$$\mathrm{PGA}^{3-} + H_2O \longrightarrow \mathrm{DHAP}^{2-} + OH^- + 1/2\ O_2.$$

Dihydroxyacetone phosphate (DHAP) is excreted by the chloroplasts [22]. The overall reaction is

$$\mathrm{glycerate}^- + HPO_4{}^{2-} \longrightarrow \mathrm{DHAP}^{2-} + OH^- + 1/2\ O_2.$$

It can be used to determine the ratio of coupling between ATP formation and generation of reductant in intact chloroplasts. If two molecules of ATP were made in intact chloroplasts in the light during transfer of two electrons to NADP as suggested by direct measurements of coupling in broken chloroplasts [7–10], the quantum requirement of glycerate reduction should not be higher than that of phosphoglycerate reduction. It should in fact be lower. Glycerate reduction needs formation of ATP and NADPH in a ratio of 2/1. In contrast, during reduction of phosphoglycerate, ATP and NADPH are consumed in a ratio of 1/1. If the ATP/2e ratio were 2/1, ATP should accumulate during phosphoglycerate reduction and should decrease the quantum efficiency by control of electron flow [9, 14]. Glycerate reduction should thus show a higher quantum efficiency than phosphoglycerate reduction. However, this is not observed. Fig. 1 shows the number of 674 nm quanta, required

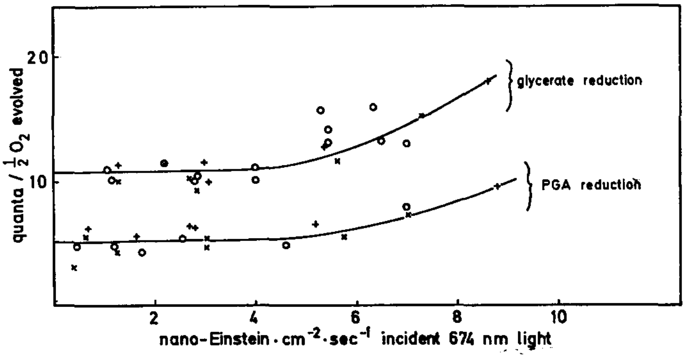

Fig. 1. Quantum requirements of glycerate- and 3-phosphoglycerate-dependent oxygen evolution of intact spinach chloroplasts at pH 7.6 as a function of the intensity of 674 nm light in 3 different experiments.

to evolve half a molecule of oxygen during phosphoglycerate and glycerate reduction at pH 7.6 as a function of light intensity. As long as light intensities and not enzyme levels were rate-limiting, glycerate reduction needed twice as many quanta as phosphoglycerate reduction in these experiments. At first sight this suggests an ATP/2e ratio close to 1 rather than of two: The most significant difference in the two reactions is the doubled ATP requirement for glycerate reduction. Doubling of the quantum requirement can easily be understood, if the coupling ratio were 1/1. An extra number of quanta equal to that required for production of 1 NADPH and 1 ATP would then be needed to synthesize the extra ATP.

However, the situation is in fact more complex. After phosphorylation of glycerate not all the formed phosphoglycerate is reduced to triosephosphate and gives rise to oxygen evolution. Part of the phosphoglycerate is excreted by the chloroplasts. The real ATP consumption during glycerate-dependent oxygen evolution is therefore higher than indicated by the stoichiometry of the reaction. It can be determined by measuring the reaction products of [^{14}C]glycerate reduction.

Fig. 2 shows product formation from [^{14}C]glycerate at pH 7.6 and 7.0 under illumination with 4.3 W · m^{-2} 674 nm light. At this intensity the reaction still increases linearly with light intensity. After turning on the light oxygen evolution starts

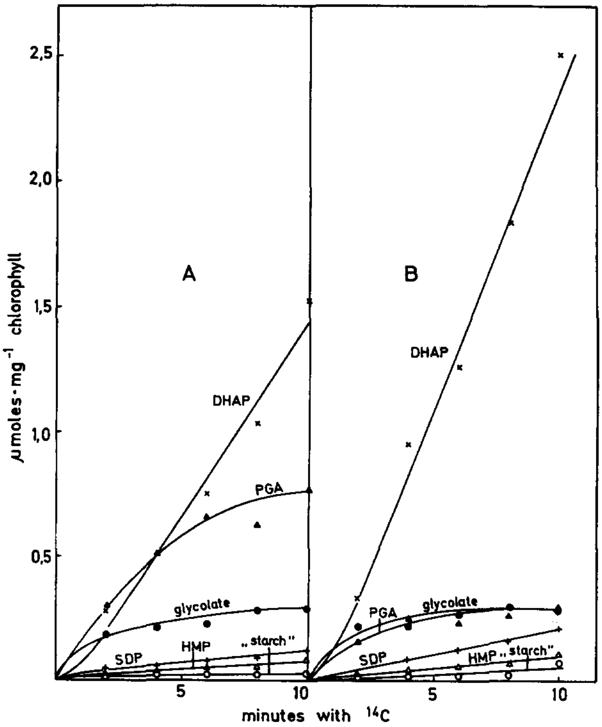

Fig. 2. Products formed by intact spinach chloroplasts from D,L-[^{14}C]glycerate as a function of reaction time. Intensity of 674 nm light 4.3 W · m^{-2}. "Starch" is expressed in hexose units. DHAP, dihydroxyacetone phosphate; PGA, phosphoglycerate; SDP, sugar diphosphates; HMP, hexose and sedoheptulose monophosphates A, pH of reaction medium 7.6; B, pH of reaction medium 7.0.

slowly and then accelerates to a steady rate [21]. During the initial lag phosphoglycerate builds up.

The later stages of the reaction are characterized by the accumulation of reduced reaction products such as dihydroxyacetone phosphate and, to a lesser extent, sugar mono- and diphosphates. There is also some formation of glycolate. It is known that oxygen is consumed during glycolate formation. Glycolate formation therefore decreases the quantum efficiency of oxygen evolution during glycerate reduction. However, similar glycolate formation has been observed during reduction of ^{14}C-labeled phosphoglycerate and of glycerate. Since the argument concerning coupling of phosphorylation to electron flow in intact chloroplasts rests on a comparison of glycerate and phosphoglycerate reduction, the effect of some glycolate formation on the quantum efficiencies of both reactions has been neglected. This is also justified from the observation that glycolate formation leveled off after about 2 min of illumination (Fig. 2). Oxygen evolution was determined only after it had attained a steady rate after several minutes in the light.

During glycerate reduction the extent of formation of phosphoglycerate, which is not subsequently reduced, is important, because phosphorylation of glycerate to phosphoglycerate consumes quanta without contributing to oxygen evolution. Fig. 2 shows that at the same light intensity more phosphoglycerate is formed from glycerate at pH 7.6 than at pH 7.0. For dihydroxyacetone phosphate an inverse relationship exists. The pH dependence of the ratio of reduced reaction products to phosphoglycerate is probably a consequence of the participation of protons in the reaction reducing 3-phosphoglycerate

$$PGA^{3-} + NADPH + ATP^{4-} + H^{+} \rightleftharpoons DHAP^{2-} + NADP^{+} + ADP^{3-} + HPO_4^{2-},$$

which favors formation of dihydroxyacetone phosphate at increased proton concentrations [21].

From the data of Fig. 2 it must be expected that the quantum yield of glycerate reduction is pH dependent. This is indeed the case. Fig. 3 shows the quantum require-

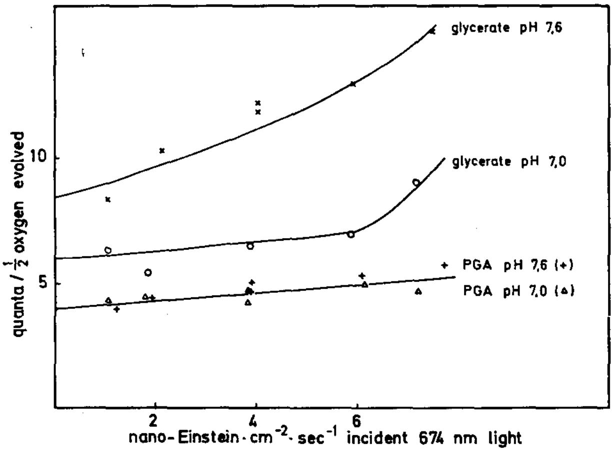

Fig. 3. Quantum requirements of glycerate- and phosphoglycerate-dependent oxygen evolution at pH 7.6 and 7.0 as a function of light intensity.

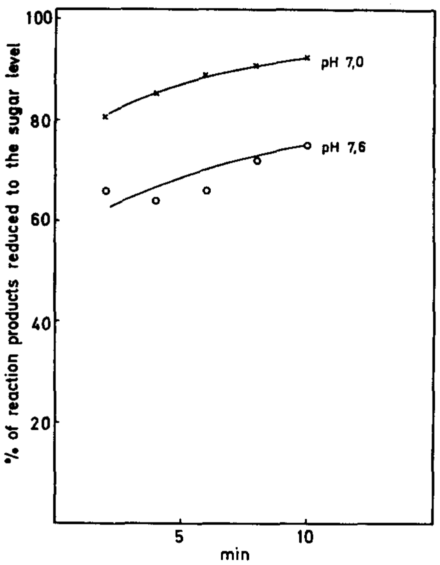

Fig. 4. The percentage of reaction products which were reduced to the sugar level during glycerate reduction in the light. Reaction products are expressed in C_3-units to permit comparison with the phosphoglycerate which was not reduced.

ments of glycerate and 3-phosphoglycerate reduction at pH 7.6 and pH 7.0 at different light intensities. Extrapolation to zero light intensity indicates a 50 % higher quantum requirement of glycerate reduction at the higher pH. In contrast, the quantum requirement of phosphoglycerate reduction was the same at pH 7.6 and 7.0. Even at the low pH the quantum requirement of glycerate reduction was higher by a factor of 1.5 than that of phosphoglycerate reduction.

Fig. 4 shows the percentage of products of glycerate reduction reduced to the sugar level. At pH 7.6 phosphoglycerate accounted for 1/2–1/3 of the reaction products, if the latter are expressed in C_3-units, at pH 7.0 for 1/7 or less. Together with the results shown in Figs 1 and 3, knowledge of the extent of phosphoglycerate accumulation during glycerate reduction facilitates calculation of the coupling ratio ATP/2e in intact chloroplasts. A premise of the calculation is that the difference in the quantum requirements of glycerate and phosphoglycerate reduction represents consumption of quanta by a photochemical reaction producing ATP without contributing to net oxygen evolution. This reaction supplies ATP needed for formation of phosphoglycerate from glycerate. Its quantum efficiency is assumed to be the same as that of ATP production during NADP reduction. The number of quanta needed for NADP reduction is, for the purpose of calculation, indicated by the quantum requirement of phosphoglycerate reduction. Table I shows for different coupling ratios (ATP/2e) a calculation of expected ratios of the quantum requirements of glycerate and phosphoglycerate reduction without and with correction for the phosphoglycerate accumulating during glycerate reduction. In the experiments shown in Figs 1 and 3 for reduction at pH 7.6 the ratios of quantum requirements for glycerate reduction to those for phosphoglycerate reduction were close to 2. Since the ratio of phosphoglycerate to reduced products was about 1/2–1/3 in these experiments,

TABLE I

RELATION BETWEEN QUANTUM REQUIREMENTS OF GLYCERATE AND PHOSPHOGLYCERATE REDUCTION AND DIFFERENT COUPLING RATIOS ATP/2e

ATP/2e	Expected ratio	$\frac{\text{Quantum requirement of glycerate reduction}}{\text{Quantum requirement of phosphoglycerate reduction}}$		
	(a) Uncorrected for phosphoglycerate formed and not reduced during glycerate reduction	(b) Corrected for a ratio between phosphoglycerate and reduced products of glycerate reduction (as expressed in C_3-units)		
		1/2	1/3	1/10
1	≈2	2.5	2.3	2.1
1.2	1.7	2.1	1.95	1.7
1.3	1.5	1.9	1.8	1.6
1.5	1.3	1.7	1.55	1.4
1.8	1.1	1.4	1.3	1.2
2.0	1 or smaller	1.25*	1.2*	1.1*

* Or smaller, depending on the extent of control of electron flow by high ATP/ADP ratios.

this corresponds to an ATP/2e ratio of about 1.2–1.3 as indicated in Table I. For reduction at pH 7.0 (Fig. 3) a ratio of the quantum requirements of about 1.5 indicates in rather close agreement a coupling ratio of 1.3–1.4 (Table I). Such coupling ratios are consistent with observations showing an ATP limitation of CO_2 reduction by intact chloroplasts [5] which is believed to require ATP and NADPH at a ratio of 1.5.

In a few experiments measured ratios of quantum requirements for glycerate and phosphoglycerate reduction indicated coupling in excess of 1.3–1.4 ATP/2e (not shown). However, no results were obtained which could be interpreted as showing coupling ratios close to 2. Such ratios have been observed by Hall in carefully prepared broken chloroplasts [9, 24]. It should be emphasized that in view of the high ATP requirement of glycerate reduction, which keeps ATP/ADP ratios low during the reaction in rate-limiting light, calculated coupling ratios probably indicate the maximal coupling possible in intact isolated chloroplasts.

3. *Oxidation of external NADH by intact chloroplasts during phosphoglycerate reduction as mediated by the malate shuttle*

Intact chloroplasts do not take up external pyridine nucleotides. However, their envelopes contain a transfer system for malate and oxaloacetate [25, 26]. Since chloroplasts contain malate dehydrogenase [27, 28], a cyclic transfer of malate and oxaloacetate can connect internal and external pyridine nucleotides. Export of reducing equivalents through a malate oxaloacetate shuttle has in fact been observed on illuminating intact chloroplasts [17, 28]. Continuous export is only possible as long as there is a gradient in the free energy of internal and external pyridine nucleotide systems. Conversely, import of reducing equivalents, i.e. oxidation of external pyridine nucleotides by intact chloroplasts, requires a sink for reducing equivalents inside the chloroplasts. In the presence of 3-phosphoglycerate chloroplasts

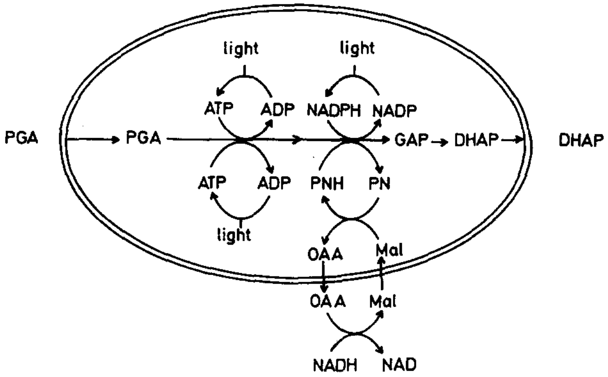

Fig. 5. Schematic representation of the oxidation of external NADH as mediated by the malate shuttle during phosphoglycerate reduction by intact chloroplasts in the light. The chloroplast envelope is impermeable to pyridine nucleotides. PGA, phosphoglycerate; GAP, glyceraldehyde phosphate; DHAP, dihydroxyacetone phosphate; PN and PNH oxidized and reduced pyridine nucleotide; OAA, oxaloacetate; Mal, malate.

no longer export reducing equivalents in the light. The direction of the reaction is reversed and external pyridine nucleotide is oxidized [28]. Reducing equivalents are taken up and are used to drive reduction of phosphoglycerate inside the chloroplasts (Fig. 5). Since this reaction requires ATP and reduced pyridine nucleotide in a 1/1 ratio, import of reducing equivalents is evidence that chloroplasts produce more ATP than reduced pyridine nucleotide. This is certainly not surprising as long as light is not ratelimiting for phosphoglycerate reduction, because ATP may then be produced in different and competing electron transport reactions, e.g. by pseudocyclic or cyclic electron flow. However, under rate-limiting light competing reactions are excluded in the presence of an electron sink such as phosphoglycerate. This is indicated by the low quantum requirement of phosphoglycerate reduction (Figs 1 and 3). Light-dependent oxidation of external pyridine nucleotide by intact chloroplasts under low light is therefore direct evidence for ATP/2e ratios higher than 1.

Fig. 6, I shows reduction of external NAD by intact chloroplasts which is mediated by the malate shuttle. This reduction was saturated at much lower light intensities (approx. 3 W · m^{-2}) than reduction of oxaloacetate (cf. Fig. 9) indicating a rate-limitation by metabolite transfer across the chloroplast envelope. In agreement with this, the rate of external NAD reduction was reduced by decreasing the redox gradient between chloroplasts and external medium, i.e. by increasing the external NADH concentration. In the presence of 3-phosphoglycerate, whose reduction is strictly light-limited at the indicated intensity, reduction of external NAD was replaced by oxidation (Fig. 6, II). The oxidation rate was also a function of the redox-gradient and was increased, when the external NADH concentration was increased (Fig. 6, III).

Under low light intensities, which do not saturate oxidation of external NADH by the malate shuttle, a comparison of the rate of phosphoglycerate reduction with that of phosphoglycerate-dependent oxidation of external NADH permits an estimation of coupling ratios. Premise of this calculation is that under these conditions the availability of ATP inside the chloroplasts determines oxidation of

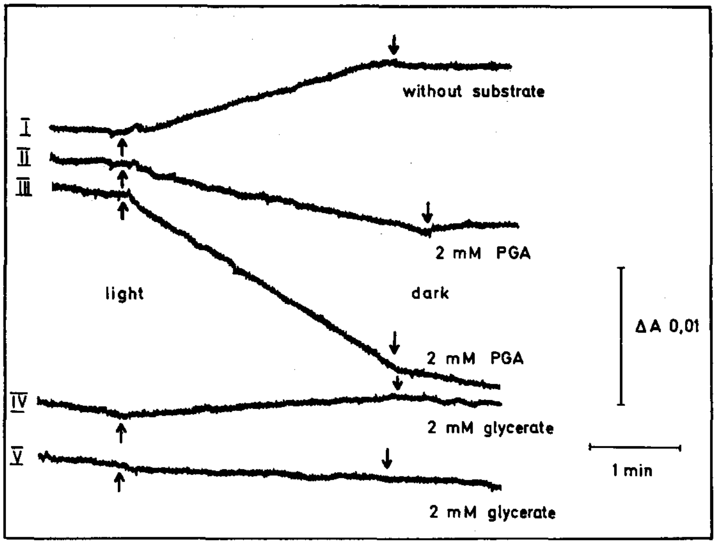

Fig. 6. Reduction or oxidation of external NAD(H) by intact spinach chloroplasts as mediated by the malate shuttle in the presence or absence of substrates. Traces show 340 nm absorption. Positive deflection caused by illumination indicates NAD reduction in the external medium, negative deflection NADH oxidation. Intensity of 674 nm light was 3.6 W · m^{-2}, light path 0.5 cm. 70 % of the chloroplasts in the preparation were intact. Traces I, II, IV: 2.5 % of the NAD in the external medium was reduced at the beginning of the experiment. Traces III, V: 22 % of the NAD in the external medium was reduced at the beginning of the experiment.

TABLE II

RATE OF PHOSPHOGLYCERATE REDUCTION BY INTACT CHLOROPLASTS AS MEASURED BY LIGHT-DEPENDENT OXYGEN EVOLUTION AND RATE OF OXIDATION OF EXTERNAL NADH THROUGH THE MALATE SHUTTLE AS SHOWN BY CHANGES IN 340 nm ABSORPTION

Coupling ratios were calculated under the assumption that the extent of indirect NADH oxidation by the chloroplasts was determined by the availability of ATP inside the chloroplasts.

Intensity of 674 nm light (W · m^{-2})	Phosphoglycerate reduction (μmoles · mg^{-1} chlorophyll · h^{-1})	Light-dependent oxidation of external NADH in p resence of 2mM phosphoglycerate (μmoles · mg^{-1} chlorophyll · h^{-1})	Calculated coupling ratio
0.85	4.8	1	1.2
0.875	4.9	0.7	1.15
1.4	7.9	2.8	1.35
2.3	13	2.2	1.15
2.5	14	2.4	1.15

external reducing equivalents. Coupling ratios calculated on this basis (Table II) agree reasonably well with coupling ratios calculated from the quantum requirements of glycerate and phosphoglycerate reduction (see above).

As should be expected, no import of reducing equivalents was possible during glycerate reduction in rate-limiting light, since no excess of ATP was available (Fig. 6, V). There was even some export of reducing equivalents at low concentrations of external NADH indicating excess of reducing equivalents inside the chloroplasts (Fig. 6, IV).

Fig. 7 shows in another experiment export of reducing equivalents during photosynthetic CO_2 reduction by intact chloroplasts. In contrast to the observations made with phosphoglycerate (Fig. 6) there was little influence of bicarbonate on the rate of export. The results may be taken as another indication that ATP is rate-limiting in CO_2 reduction and that coupling ratios are therefore likely to be below 1.5.

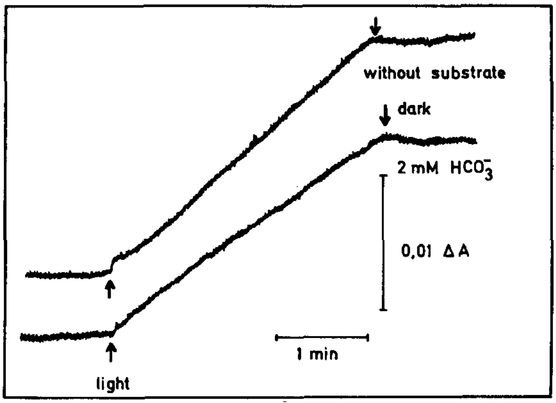

Fig. 7. Reduction of external NAD by intact chloroplasts through the malate shuttle in the presence or absence of 2 mM HCO_3^-. Intensity of red light was 4.8 W · m^{-2}. For further description see legend to Fig. 6. 78 % of the chloroplasts in the preparation had intact envelopes.

4. Reduction of oxaloacetate by intact chloroplasts

Broken chloroplasts reduce added electron acceptors in the light even in the absence of ADP and inorganic phosphate or in the presence of ATP, though at reduced rates. Electron transport under these conditions has been called non-phosphorylating or basal electron transport and has been distinguished from coupled electron transport, which is accompanied by phosphorylation [7, 29]. The question has not been resolved as to whether it is a physiological reaction and has to be attributed to a special non-phosphorylating electron transport pathway, or whether it is caused by pathological uncoupling during thylakoid isolation. Another possibility is that it simply reflects electron transport made possible even in the absence of a phosphorylating system or in the presence of ATP by "loose" or flexible coupling. It has, at any rate, often been subtracted from the electron transport rate observed in the presence of a phosphorylating system in order to calculate "true" ATP/2e ratios [7, 9, 29]. However, it is questionable whether this is justified. Fig. 8 shows oxaloacetate reduction by intact chloroplasts in the absence and in the presence of uncou-

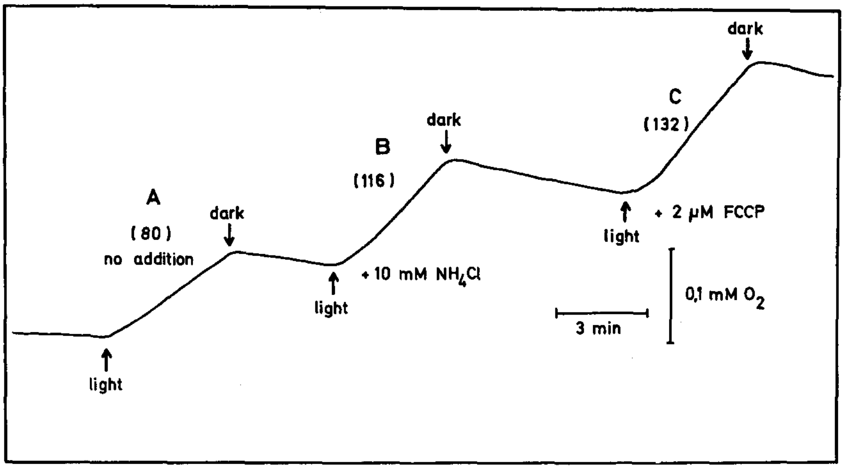

Fig. 8. Reduction of oxaloacetate (2 mM) by intact chloroplasts in the light as indicated by oxygen evolution in the presence of the photosynthesis inhibitor glyceraldehyde (10 mM). Trace shows response of the oxygen electrode. Upwards directed arrow indicates illumination, downwards directed arrow darkening. Numbers in brackets give rates of oxaloacetate reduction in μmoles · mg^{-1} chlorophyll · h^{-1}. A, without uncoupler; B, 10 mM NH_4Cl added; C, further addition of 2 μM FCCP. Intensity of red light was 250 W · m^{-2}. CO_2 reduction by the same preparation was 148 μmoles · mg^{-1} chlorophyll · h^{-1}.

plers. 10 mM glyceraldehyde was also present to inhibit any CO_2-dependent oxygen evolution. Stokes and Walker found glyceraldehyde to be a potent inhibitor of CO_2 reduction [30]. Fully coupled chloroplasts (CO_2 reduction at light saturation 148 μmoles · mg^{-1}) reduced 80 μmoles oxaloacetate · mg^{-1} chlorophyll · h^{-1}. Uncoupling by 10 mM NH_4Cl was unable to increase this rate drastically. Since 10 mM NH_4Cl does not always uncouple efficiently, as seen by incomplete inhibition of CO_2 reduction, FCCP was also added. Still the rate of oxaloacetate reduction was increased by a factor of less than 2. Phlorizin, which is considered to be an energy transfer

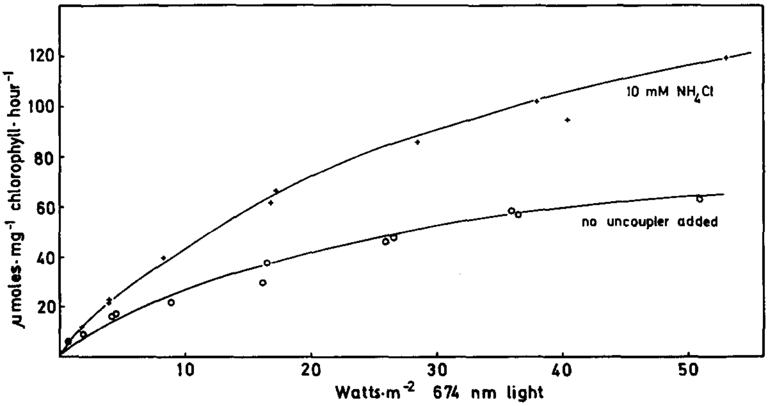

Fig. 9. Reduction of oxaloacetate by intact chloroplasts in the absence and in the presence of 10 mM NH_4Cl as a function of light intensity.

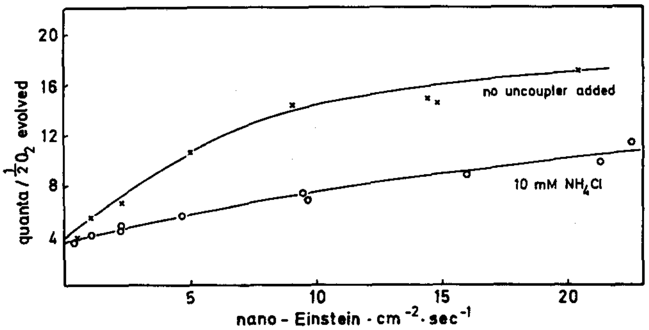

Fig. 10. Quantum requirements of oxaloacetate reduction by intact chloroplasts, in the presence and absence of 10 mM NH_4Cl, as a function of the intensity of 674 nm light.

inhibitor (ref. 29, but see ref. 31) and completely inhibited CO_2 reduction at concentration of 1 mM, failed to inhibit oxaloacetate reduction by fully coupled chloroplasts by more than 15 %. Fig. 9 shows in another experiment oxaloacetate reduction by intact chloroplasts in the absence and in the presence of 10 mM NH_4Cl, as a function of light intensity. It is again apparent, that even fully coupled chloroplasts reduce oxaloacetate efficiently, though ATP is not consumed during reduction of oxaloacetate to malate. If coupling between phoshorylation and electron flow were tight, this should not be possible. The situation becomes even clearer when quantum requirements for oxaloacetate and phosphoglycerate reduction are compared (Fig. 10, cf. Fig. 1). Extrapolation to zero light intensity yields the same "basic" quantum requirement of 4 for oxaloacetate reduction by coupled and uncoupled chloroplasts and for phosphoglycerate reduction. However, while the quantum requirement of phosphoglycerate reduction was not drastically dependent on light intensity over a limited range (Fig. 1), the quantum requirement of oxaloacetate reduction by coupled chloroplasts increased with light intensity. That this increase was due to control of electron flow by the high ATP potential is shown by decreased quantum requirements in the presence of uncouplers such as ammonium ions. The observed intensity dependence of quantum yield of coupled chloroplasts is inconsistent with the idea of a non-phosphorylating electron transport chain. Electron transfer in such a pathway should not be subject to adenylate control. Within the capacity of such a pathway quantum yields should therefore show little dependence on light intensity. This is not the case. Rather the data of Figs 8–10 indicate flexibility of coupling. Fully coupled chloroplasts require only an extra input of energy to overcome the restrictive influence of coupling as seen by the parallel course of quantum requirements of coupled and uncoupled chloroplasts at higher intensities (Fig. 10). The restriction put on electron flow by coupling appears to be a function of the physiological state of the material and has been found to vary in different preparations of intact chloroplasts. In an extreme example, the same chloroplasts which supported 172 μmoles · mg^{-1} chlorophyll · h^{-1} CO_2-dependent and 135 μmoles · mg^{-1} chlorophyll · $^{-1}$ phosphoglycerate dependent oxygen evolution, reduced 133 μmoles · mg^{-1} chlorophyll · h^{-1} oxaloacetate. No uncoupler was added to enhance reduction. Chloroplasts from other preparations were more tightly coupled. Table III compares ratios of oxaloacetate reduc-

TABLE III

RATIOS OF OXALOACETATE REDUCTION BY UNCOUPLED (10 mM NH_4Cl) AND FULLY COUPLED SPINACH CHLOROPLASTS FROM DIFFERENT LEAF MATERIAL, COMPARED WITH PHOSPHOGLYCERATE AND CO_2 REDUCTION

$W \cdot m^{-2}$	$\frac{\text{Rate of reduction of oxaloacetate by uncoupled chloroplasts}}{\text{Rate of reduction of oxaloacetate by coupled chloroplasts}}$		
	Expt a	Expt b	Expt c
50	—	2.5	1.9
40	10.5	2.5	1.8
30	8.5	2.4	1.75
20	6.5	2.1	1.7
10	4.7	1.6	1.65
5	—	1.3	1.6
2.5	—	—	1.3
	Phosphoglycerate reduction at light saturation (μmoles · mg^{-1} chlorophyll · h^{-1})		
230	132	146	158
	CO_2 reduction at light saturation (μmoles · mg^{-1} chlorophyll · h^{-1})		
230	30	80	125

tion by chloroplasts uncoupled by 10 mM NH_4Cl and by coupled chloroplasts at different light intensities. These ratios increased with light intensity. At low intensities stimulation of rates by uncoupling was slight. At intensities approaching saturation of reduction stimulation ranged from a factor of less than 2 (sometimes not more than 1.5) to more than 10. Surprisingly, CO_2 reduction rates appeared to be higher with chloroplasts, which did not show a large stimulation of oxaloacetate reduction by uncoupling (Table III).

5. Significance of the results

The results of this investigation suggest that ATP is a rate-limiting factor in photosynthesis. ATP/2e ratios were obtained under conditions of electron transport proceeding in the presence of low intrachloroplast levels of ATP and high levels of ADP (cf. ref 5). They were distinctly higher than 1, but in most cases also clearly below 1.5. Flexibility of coupling as derived from measurements of oxaloacetate reduction implies, that the extent of coupling decreases with increasing phosphorylation potential [ATP]/[ADP][P_i]. It is therefore possible that coupling ratios during CO_2 photosynthesis are even lower than indicated above. Since photosynthesis consumes ATP and NADPH at a ratio probably not far from 1.5, this raises the question of where the extra ATP required for photosynthesis comes from.

It is, on the other hand, possible to argue, that ATP/2e ratios below 1.5 as observed in this investigation may simply reflect partial damage even to intact chloroplasts during the isolation procedure, and that in vivo coupling is better so that the ATP requirements of photosynthesis are satisfied by ATP formation during electron flow to NADP. While this possibility cannot be discounted, the point to make is that in spite of low ATP/2e ratios the isolated chloroplasts are quite capable of photoreducing CO_2 at high rates. They can even reduce glycerate, whose reduction requires more ATP than that of CO_2. Obviously they have a mechanism to supply the extra ATP, which is not provided by electron flow to NADP. If isolated chloroplasts have such a mechanism, it should clearly exist also in the leaf. It would be surprising, if it had no function there.

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REFERENCES

1 Bassham, J. A. (1964) Annu. Rev. Plant Physiol. 15, 101–120
2 Avron, M. and Neumann, J. (1968) Annu. Rev. Plant Physiol. 19, 137–166
3 Schuermann, P., Buchanan, B. B. and Arnon, D. I. (1972) Biochim. Biophys. Acta 267, 111–124
4 Tanner, W., Löffler, M. and Kandler, O. (1969) Plant Physiol. 44, 422–428
5 Heber, U. (1973) Biochim. Biophys. Acta 305, 140–152
6 Heber, U. (1960) Biochim. Biophys. Acta 180, 302–319
7 Izawa, S. and Good, N. E. (1968) Biochim. Biophys. Acta 162, 380–391
8 Hall, D. O. and Evans, M. C. W. (1972) J. Subcell. Biochem. 1, 197–206
9 Reeves, S. G. and Hall, D. O. (1973) Biochim. Biophys. Acta 314, 66–78
10 West, K. R. and Wiskich, J. T. (1973) Biochim. Biophys. Acta 292, 197–205
11 Gimmler, H. (1973) Z. Pflanzenphysiol. 68, 289–307
12 Trebst, A. and Reimer, S. (1973) Biochim. Biophys. Acta 305, 129–139
13 Izawa, S., Gould, J. M., Ort, D. R., Felker, P. and Good, N. E. (1973) Biochim. Biophys. Acta 305, 119–129
14 Shavit, N. (1972) Proc. 2nd Int. Congr. on Photosynth. Res. (Forti, G., Avron, M. and Melandri, A., eds), pp. 1221–1231, Dr W. Junk N.V., The Hague
15 Jensen, R. G. and Bassham, J. A. (1966) Proc. Natl. Acad. Sci. U.S. 56, 1095–1101
16 Heber, U. and Santarius, K. A. (1970) Z. Naturforschg. 25b, 718–728
17 Heber, U. and Krause, G. H. (1972) Proc. 2nd Int. Congr. on Photosynth. Res. (Forti, G., Avron, M. and Melandri, A., eds), pp. 1023–1033, Dr W. Junk N.V., The Hague
18 Krause, G. H. (1974) Biochim. Biophys. Acta 333, 301–313
19 Kautsky, H. and Hirsch, G. (1934) Biochem. Z. 274, 422–434
20 Hill, R. and Bendall, F. (1960) Nature 186, 136–137
21 Heber, U., Kirk, M. R., Gimmler, H. and Schäfer, G. (1974) Planta, in the press
22 Walker, D. A. (1974) MTP Int. Rev. Sci. Biochem. Sect. (Northcote, D. H., ed.), Vol. 11, 1–49, Butterworth, London
23 del Campo, F. F., Ramirez, J. M. and Arnon, D. I. (1968) J. Biol. Chem. 243, 2805–2809
24 Reeves, S. G., Heathcote, P. and Hall, D. O. (1972) 6th Int. Congr. Photobiol., Bochum, Book of Abstracts (Schenck, G. O., ed.), p. 84, Max-Planck-Institut für Kohlenforschung, Mülheim
25 Heldt, H. W. and Rapley, L. (1970) FEBS Lett. 10, 143–148
26 Heldt, H. W. and Sauer, S. (1970) Biochim. Biophys. Acta 234, 83–91
27 Johnson, H. S. and Hatch, M. D. (1970) Biochem. J. 119, 273–280
28 Heber, U. and Krause, G. H. (1971) in Photosynthesis and Photorespiration (Hatch, M. D., Osmond, C. B. and Slatyer, R. O., eds), pp. 218–225, Wiley Interscience, New York
29 Izawa, S., Winget, G. D. and Good, N. E. (1966) Biochem. Biophys. Res. Commun. 22, 223–226
30 Stokes, D. M. and Walker, D. A. (1972) Biochem. J. 128, 1147–1157
31 West, J. and Hall, D. O. (1973) Biochem. Soc. Trans. 1, 887–890